Salivary biochemistry of the healthy oral ecosystem

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2017

document version
Publisher's PDF, also known as Version of record

CHAPTER

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Interindividual variation, correlations, and sex-related differences in the salivary biochemistry of young healthy adults

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European Journal of Oral Sciences 2015, 123(3): 149-157

Abstract

A cross-sectional observational study was conducted to evaluate interindividual biochemical variation in unstimulated whole saliva in a population of 268 systemically healthy young students aged 18-30 yr (150 males and 118 females) with no of apparent caries lesions or periodontal disease. Salivary flow rate, protein content, pH, buffering capacity, mucins MUC5B and MUC7, albumin, secretory-IgA, cystatin S, lactoferrin, chitinase, amylase, lysozyme and proteases were measured using Enzyme-Linked Immunosorbent Assays (ELISA) and enzymatic activity assays. Significant differences were found between males and females. Salivary pH, buffering capacity, protein content, MUC5B, secretory-IgA, and chitinase activity were all lower in females compared to males, while MUC7 and lysozyme activity were higher in females ($p < 0.05$). There was no significant difference between sexes in salivary flow rate, albumin, cystatin S, amylase and protease activity. Principal Component Analysis (PCA) and Spectral Clustering (SC) were used to assess inter-variable relationships within the dataset and to identify subgroups. SC analysis identified 2 clusters of participants, which were subsequently described. This study provides a comprehensive overview of the distribution and interrelations of a set of important salivary biochemical variables in the young systemically healthy young adult population screened for the absence of apparent caries lesions and periodontal disease. It highlights significant gender differences in salivary biochemistry.

Introduction

Saliva is crucial for the maintenance of oral health. It initiates the digestion of carbohydrates, provides lubrication as a countermeasure to tooth wear, stabilizes oral pH and helps the re-mineralization of enamel [1, 2]. Saliva prevents pathogenic microorganisms from colonizing the mouth and subsequently causing disease [2-4]. It aggregates and clears planktonic bacteria, whereas the protective coating it forms on tooth surfaces may serve as the foundation for oral biofilm (plaque) formation [1, 3]. Many of the individual proteins and biochemical processes that mediate these functions have been thoroughly studied [1]. However, salivary components may perform multiple, sometimes overlapping tasks and form a functional network characterized by interactions and redundancies [5-7]. No single salivary constituent has a decisive effect on oral health by itself. It is rather the properties of the salivary functional network as a whole that determine whether the oral ecosystem maintains homeostasis or shifts towards a disease state [2].

The starting point in understanding the action of saliva as a biological system in relation to oral health is to investigate what the constitution of this system is when it is functioning properly: i.e. the salivary biochemistry of individuals with healthy mouths. Yet, despite extensive salivary research over the last decades, this work has not resulted in a clear picture [8]. There are many factors that can influence the results of clinical studies focusing on saliva analysis. This makes it difficult to compare and compile the results of separate studies into an integrated model. Age and sex differences of the participants, (oral) health status, medication, physical activity and level of oral self-care can all influence salivary composition [2, 9, 10]. Even the circadian cycle - and therefore the time of day when saliva is collected - has a significant effect [11, 12]. Sample processing and storage conditions can also affect results. Saliva is predisposed to proteolysis and salivary protein aggregation may occur during sample freezing and thawing [13, 14]. Centrifugation, often used to remove insoluble aggregates and oral bacteria, can also remove some of the salivary proteins to a varying extent [15].

The primary aim of this study was to provide a detailed description of the distribution, interindividual variation and interrelations of a broad set of salivary proteins and other biochemical variables in a systemically healthy young adult population. A secondary aim was to identify and visualize subgroups of individuals based on their salivary protein biochemistry. Unstimulated whole saliva was collected from 268 young adults screened for good oral health. The levels of ten major salivary proteins and enzymes with known relevance for oral health were measured, as well as salivary flow rate, pH, buffered pH and total protein content.

Materials and Methods

Clinical study structure, study population and exclusion criteria

The study was carried out within the framework of the Top Institute Food and Nutrition project "Estimating the boundaries for a healthy oral ecosystem in young individuals".

Whole unstimulated saliva was collected in a cross-sectional single-center observational clinical study at the Academic Center for Dentistry Amsterdam (ACTA). The study population comprised a convenience sample of systemically healthy young adults aged 18-30 yr old without periodontitis. Participants were students of universities and colleges in and around Amsterdam, The Netherlands. They were invited for screening when they had visited their general dentist the previous year and had been considered to be without oral or dental problems. The volunteers were screened for suitability according to the criteria as proposed for the Dutch Periodontal Screening Index (DPSI) [16]. They were included if they had a DPSI $\leq$ 3-. The exclusion criteria were: presence of overt dental caries, inter-proximal restorations between the first and second, or second and third upper molars, apparent oral lesions, a habit of smoking, infections, recent use of antibiotics, use of anti-inflammatory drugs or other prescribed medication which could interfere with the outcome of this study (except for oral contraceptives). Participants were instructed not to eat, drink, chew gum or perform strenuous physical exercise prior to the appointment, and not to brush their teeth in the morning of the appointment or the evening before.

The menstrual cycle phase (menstrual, proliferative, follicular, luteal or secretory) was noted for female participants at the date of saliva sampling.

The study was conducted in accordance with the Declaration of Helsinki (2008) of the World Medical Association and approximating Good Clinical Practice guidelines. The study protocol was reviewed and approved by the Medical Ethics Committee of the Academic Medical Centre of Amsterdam (2012_210#B2012406) and registered at the Dutch Trial Register (NTR3649). All participants signed an informed consent form.

Unstimulated saliva sampling and pH measurement

All saliva samples were collected between 9 and 10 a.m. Participants were instructed to allow saliva to accumulate in the floor of the mouth without stimulation such as orofacial movements and to spit at 30 s intervals into pre-weighed 30-ml polypropylene tubes (Sterilin, Newport, U.K.) which were kept on ice. The collection period was 5 min. The tubes containing the unstimulated saliva samples were weighed and salivary flow rate was calculated assuming a saliva density of 1.0 g ml^{-1}. Output was calculated as flow rate

multiplied by protein concentration. Samples were homogenized by vortexing for 20 s. Salivary pH was measured immediately after acquisition and homogenizing using a Eutech pH 5+ microelectrode pH meter (ThermoScientific, West Palm Beach, U.S.A.). The pH buffering capacity of saliva was assayed as follows: 20 µl of sample were mixed with 40 µl of 0.005 N HCl, briefly vortexed and incubated at room temperature for 30 s before measurement of the buffered pH [17]. Samples were clarified by centrifugation for 10 min at 4°C and 10,000 *g* to remove epithelial cell debris, bacteria and food residues. The resulting clarified saliva was diluted 1:1 with a 500 mM NaCl solution to a final concentration of 250 mM NaCl, aliquoted and stored at -80°C. It had been verified beforehand that diluting saliva with a NaCl solution would not interfere with ELISAs or enzyme activity assays. The dilution prevented protein aggregation and precipitation during saliva freezing and storage and lowered viscosity allowing for more precise sample manipulation and improved reproducibility (PRODAN *et al.* manuscript in preparation). A sufficient number of aliquots of each sample were produced to avoid exposing samples to multiple freezing and thawing cycles.

Total protein content determination

Total protein content was measured with a Pierce BCA Protein Assay Kit (ThermoScientific, West Palm Beach, USA) in polystyrene 96-well microplates (Greiner Bio-One, Frieckenhausen, Germany) according to the manufacturer's specifications. Bovine serum albumin (BSA) was used as a standard. Optical readouts for the BCA assay and for all ELISAs performed in this study were obtained with a Multiscan FC microplate photometer (ThermoScientific, West Palm Beach, U.S.A.).

Mucins MUC5B and MUC7 quantification

High-binding polystyrene 96-well microplates (Greiner Bio-One, Frieckenhausen, Germany) were used for all ELISAs. For mucin ELISA, 100 µml of each saliva sample was diluted 1:100 and coated onto microplates by incubating overnight at 4°C. Each sample was 2-fold serially-diluted in coating buffer (0.1 M Na_2CO_3, pH = 9.6). MUC5B levels were then determined using mAb F2, as previously described [18]. MUC7 levels were determined using a polyclonal rabbit anti-MUC7 Ab [19]. All microplates contained a reference sample consisting of pooled saliva from 10 volunteers. The concentration of MUC5B and MUC7, respectively, in the reference sample was expressed as 1 Arbitrary Unit (AU) ml^{-1}.

Albumin quantification

Microplates were coated with a rabbit polyclonal anti-human albumin Ab (Sigma-Aldrich, St. Louis, U.S.A.) overnight at room temperature [9]. Samples were subsequently 2-fold serially-diluted and incubated for 2 h at 37°C. Captured salivary albumin was detected using a HRP-conjugated rabbit anti-human albumin Ab (GeneTex, Inc., Irvine, CA, U.S.A.). Human serum albumin (Sigma-Aldrich, St. Louis, U.S.A.) was used as a standard.

Lactoferrin quantification

Microplates were coated with polyclonal rabbit anti-human lactoferrin Ab (Sigma-Aldrich, St. Louis, U.S.A.). Purified human lactoferrin (Sigma-Aldrich, St. Louis, U.S.A.) was used as a standard. Captured lactoferrin was assayed with a HRP-conjugated rabbit anti-human lactoferrin Ab (RayBiotech, Norcross, GA, U.S.A.) [20].

Secretory-IgA quantification

Microplates were coated overnight at room temperature with monoclonal rabbit anti-human secretory-IgA Ab (Sigma-Aldrich, St. Louis, U.S.A.). The microplates were then incubated with the saliva samples and a standard of purified human secretory-IgA (Nordic-MUbio, Susteren, The Netherlands) for 2 h at 37°C. Captured secretory-IgA was detected with HRP-conjugated goat anti-human IgA Ab (Sigma-Aldrich, St. Louis, U.S.A.) [21].

Cystatin S quantification

Samples were coated directly onto microplates overnight at 4°C together with a pooled saliva reference sample. Cystatin S was detected with a monoclonal mouse anti-human cystatin S Ab developed and characterized as described by HENSKENS *et al.* [22]. Captured antibodies were detected with polyclonal HRP-conjugated rabbit anti-mouse Ab (Sigma-Aldrich, St. Louis, U.S.A.).

Enzymatic activity assays

Black 96-well polypropylene microplates (Greiner Bio-One, Frieckenhausen, Germany) were used for all fluorescence-based enzymatic activity assays. Amylase activity was measured with an EnzChek Ultra Amylase Assay kit (ThermoScientific, West Palm Beach, U.S.A.) according to the manufacturer's specifications. Chitinase activity was quantified by adding 50 µl saliva to a substrate solution of 4-methylumbelliferyl β-D-N,N′,N′′-

triacetylchitotrioside (Sigma-Aldrich, St. Louis, U.S.A.) to a final reaction volume of 200 µl and a substrate concentration of 40 nM. The increase in fluorescence was subsequently acquired for 15 min at 37°C [23]. Lysozyme activity was measured using an EnzChek Lysozyme Activity kit (ThermoScientific, West Palm Beach, U.S.A.) according to manufacturer's specifications.

Protease activity was measured based on the cleavage of two Fluorescence Resonance Energy Transfer (FRET) substrates designated BikKam9 and BikKam15, developed and previously described in detail by KAMAN *et al.* [24]. The Bikkam9 substrate is highly specific for proteases of the periodontal pathogen *Porphyromonas gingivalis,* whereas BikKam15 was used to assess the overall protease activity in saliva. Fluorescence was recorded at 2 min intervals in a BMG Fluostar Galaxy microplate reader (MTX Lab Systems, Inc., Vienna, U.S.A.).

All ELISAs and enzymatic activity assays were performed in duplicate.

Data analysis and statistical methods

The data were statistically analyzed and Principal Component Analysis (PCA) was performed using SPSS 21.0 software (IBM, Armonk, NY, U.S.A.). The normality of the distributions of salivary biochemical variables was assessed with one-sample Kolmogorov-Smirnov tests as well as by visual examination of the individual variable distribution histograms. In case of a non-normal distribution non-parametric tests were chosen for subsequent analysis. Spearman's test was used for correlations and Mann-Whitney U tests were used to compare distributions. The statistical significance level used was 0.05. The Benjamini-Hochberg False Discovery Rate (FDR) was used to correct for multiple comparisons [25]. The FDR was set at 0.05.

Spectral Clustering

Spectral Clustering (SC) analysis was performed on the final dataset containing the data from all biochemical assays. SC was performed in open-source Python 2.7 using the neighborhood co-regularized SC algorithm developed by TSIVTSIVADZE *et al*. [26] based on the SC method published by VON LUXBURG [27]. For this purpose, the data from each biochemical assay were scaled to equal ranges and a similarity matrix was calculated based on the Euclidean distances between each pair of participants (i.e. on the similarity of the overall salivary biochemistry of each pair of participants). A co-occurrence matrix was subsequently calculated based on the clustering results, quantifying the tendency of any two participants to fall within the same cluster over many k-means clusterings using varying parameters. Matlab R2012b (MathWorks, Natick, MA, U.S.A) was used to produce the co-occurrence clustering plots. After visual examination of the SC plots the number of clusters

was determined and participants were mathematically assigned to the clusters using a probabilistic decomposition algorithm [28].

Results

Prior to the study, 336 potential participants were screened at the clinic in a separate session. Of these, 46 (23 males and 23 females) were excluded based on the inclusion and exclusion criteria (14 subjects had interproximal dental restorations, 13 used medications which could interfere with the outcome of this study, nine subjects showed evidence of overt caries, four subjects had a DPSI score of ≥ 3+, three subjects did not visited their general dentist within the previous year, one presented an oral piercing, one subject was a smoker and one had a schedule conflict), 10 took part in a pilot study (not included in the final data) and 12 dropped out (11 due to schedule conflicts and one reported pregnancy). In total, 268 participants completed the study (150 males, 118 females), with a mean age of 22.6 yr (on the day of their appointment) and a range of 18-32 yr. Two of the subjects were unable to provide any saliva during the sampling period. Data obtained from the remaining 266 subjects (148 male and 118 female) is presented in this article. Due to insufficient amounts of saliva obtained from some participants several assays could not be performed on all samples. Across the whole study, the average percentage of samples available for analysis for an individual assay was 97%, with a minimum of 90% for the MUC7 measurement.

Table 1 provides a summary of the mean outcomes of the salivary biochemistry analyses. With the exception of salivary pH, the distributions of all biochemical variables showed significant deviations from normality. This inference was supported by visual inspection of the distribution histograms of each variable. There was no measurable cleavage of the Bikkam9 protease substrate by proteases specific to *P. gingivalis* in any of the samples. Therefore, only the results of the general salivary protease activity assay are presented.

Table 1. Salivary biochemistry results for the study population.

Variable	Unit	Median	Mean ± SD (N)*	95% Confidence Interval for Mean
pH		7.05	7.04 ± 0.28 (256)	7.00 - 7.07
Buffered pH		6.30	6.16 ± 0.63 (256)	6.09 - 6.24
Flow rate	ml min^{-1}	0.34	0.38 ± 0.22 (266)	0.37 - 0.41
Protein content	mg ml^{-1}	0.92	0.99 ± 0.34 (264)	0.95 - 1.03
MUC5B	AU ml^{-1}	1.0	1.2 ± 1.1 (265)	1.2 - 1.4
MUC7	AU ml^{-1}	3.8	5.8 ± 6.3 (241)	5.0 - 6.6
Albumin	µg ml^{-1}	20	37 ± 54 (266)	30 - 43
Secretory-IgA	µg ml^{-1}	261	305 ± 186 (266)	283 - 328
Cystatin S	AU ml^{-1}	1.0	2.5 ± 3.6 (265)	2.1 - 2.9
Lactoferrin	µg ml^{-1}	0.9	1.0 ± 0.7 (246)	1.0 - 1.1
Chitinase activity	AU ml^{-1}	19	32 ± 40 (263)	27 - 37
Amylase activity	U ml^{-1}	27	41 ± 34 (266)	37 - 45
Protease activity	AU ml^{-1}	44	47 ± 26 (259)	44 - 50
Lysozyme activity	U ml^{-1}	1505	1650 ± 1260 (246)	1492 - 1809

*N is the number of samples assayed for the respective variable out of the total sample population of 268 participants.

Principal Component Analysis (PCA) performed on the salivary biochemistry dataset indicated that salivary total protein content was the main contributor to the 1st principal component (PC1) (Fig. 1). Indeed, most of the salivary proteins assayed correlated to some extent with total protein content, as shown in Table 2. However, the strength of these correlations varied from $\rho = 0.73$ for secretory-IgA, to a not significant correlation for cystatin S (Table 2). There was a significant negative correlation between salivary flow rate and total protein content ($\rho = -0.47$, $p < 0.001$). These two relationships were mirrored in the negative correlations between flow rate and most individual protein concentrations. With the exception of MUC7, the negative correlations between individual proteins and flow rates were smaller than the corresponding positive correlations between the respective proteins and total protein content (Table 2).

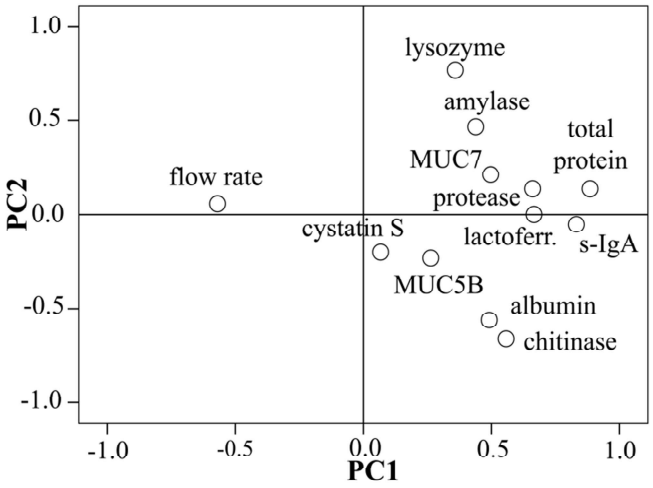

Figure 1. PCA loadings plot. PC1 and PC2 account for 35% and 14% of total variance, respectively (49% cumulative).

Table 2. Spearman's rank correlation coefficients between individual salivary proteins, total protein content and flow rate, respectively. Variables are arranged in decreasing order of the strength of their correlation with total protein content.

	Total protein content	Flow rate
Secretory-IgA	0.73	-0.64
Lactoferrin	0.61	-0.34
Albumin	0.52	-0.38
Amylase activity	0.48	NS*
Protease activity	0.44	-0.31
MUC5B	0.34	-0.21
Lysozyme activity	0.32	NS
Chitinase activity	0.29	-0.24
MUC7	0.29	-0.38
Cystatin S	NS	NS

*NS – not significant, $p > 0.05$. All other correlations shown have $p < 0.001$.

To assess intrinsic relationships between salivary proteins independent of the effect of total protein content and flow rate, a partial correlation analysis was performed in which the two aforementioned variables were controlled for. This revealed positive partial correlations between MUC7 and lysozyme ($\rho = 0.43$), and albumin and chitinase ($\rho = 0.55$), as well as a negative correlation between chitinase and lysozyme ($\rho = -0.46$) (all with $p < 0.001$). These relationships are supported by the PCA loadings plot (Fig. 1). Albumin and chitinase are grouped close together, whereas chitinase and lysozyme are situated on opposite regions of the plot, separated by the 2nd principal component. There was also a negative correlation between lysozyme activity and salivary pH ($\rho = -0.44$, $p < 0.001$), illustrated in Fig. 2. This correlation was maintained when it was controlled for the effects of flow rate and protein content ($\rho = -0.48$, $p < 0.001$).

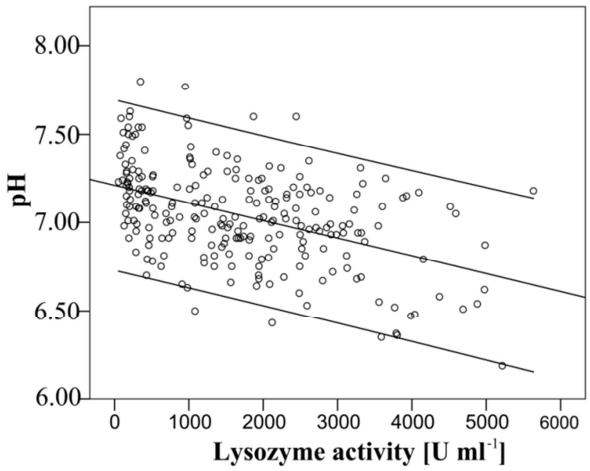

Figure 2. Scatter plot of lysozyme activity versus salivary pH. Upper and lower lines indicate 95% confidence interval.

Several significant differences were observed when the distributions of salivary components in males were compared to those of females (Tables 3 and 4). Marked differences were seen in the distribution of salivary pH and buffered pH values (Fig. 3). Salivary pH values were normally distributed for both sexes, but the mean salivary pH of females was 0.23 pH units lower compared to males, while the mean buffered pH was 0.50 units lower. Also, more females had buffered pH values in the extreme low range: 15 females had buffered pH below 5.0, compared to only 4 males. There were no significant differences in salivary biochemistry between groups of women at different phases of their menstrual cycle, when compared using a one-way ANOVA test.

Table 3. Salivary variables stratified according to gender. Percentage differences are shown for variables with distributions in males found to be significantly different compared to females using a Mann-Whitney U test.

	Unit	Median		Mean ± SE[1]		Percentage difference	*p*-value
		Male	Female	Male	Female		
pH		7.13	6.94	7.13 ± 0.02	6.90 ± 0.02	0.23*	<0.001
Buffered pH		6.51	6.07	6.40 ± 0.04	5.90 ± 0.06	0.50*	<0.001
Flow rate	ml min^{-1}	0.36	0.35	0.40 ± 0.03	0.36 ± 0.03	NS	0.121
Protein content	mg ml^{-1}	1.00	0.82	1.05 ± 0.03	0.91 ± 0.03	15%*	<0.001
MUC5B	AU ml^{-1}	1.15	0.92	1.40 ± 0.08	1.22 ± 0.11	15%*	0.028
MUC7	AU ml^{-1}	3.3	4.1	5.41 ± 0.55	6.36 ± 0.61	18%†	0.020
Albumin	µg ml^{-1}	22.5	16.0	36.4 ± 4.0	37.8 ± 5.6	NS	0.051
Lactoferrin	µg ml^{-1}	0.97	0.74	1.09 ± 0.05	0.98 ± 0.07	NS‡	0.042
Cystatin S	AU ml^{-1}	1.1	0.8	2.64 ± 0.31	2.28 ± 0.31	NS	0.113
S-IgA	µg ml^{-1}	287	229	324 ± 14	282 ± 18	15%*	0.002
Amylase activity	U ml^{-1}	29.5	23.8	44.4 ± 3.0	37.2 ± 2.8	NS	0.199
Chitinase activity	mU ml^{-1}	22.6	17.2	38.7 ± 3.8	24.6 ± 2.6	57%*	0.019
Protease activity	AU ml^{-1}	47.2	42.0	48.2 ± 2.3	47.1 ± 2.3	NS	0.813
Lysozyme activity	U ml^{-1}	1342	1660	1541 ± 112	1794 ± 112	16%†	0.026

[1]SE - standard error.

* Distributions significantly different, with mean higher in males.

† Distributions significantly different, with mean higher in females.

‡ Although $p < 0.05$, not significant after FDR adjustment.

Table 4. Salivary variables stratified according to gender, results expressed in units of output per min (calculated as flow rate x concentration). Percentage differences are shown for variables with distributions in males found to be significantly different compared to females using a Mann-Whitney U test.

	Unit	Median		Mean ± SE[1]		Percentage difference	*p*-value
		Male	Female	Male	Female		
Protein content	mg min^{-1}	0.35	0.29	0.39 ± 0.01	0.30 ± 0.01	26%*	<0.001
MUC5B	AU min^{-1}	0.39	0.27	0.48 ± 0.03	0.40 ± 0.04	19%*	0.02
MUC7	AU min^{-1}	1.30	1.51	1.83 ± 0.21	2.05 ± 0.20	NS	0.44
Albumin	µg min^{-1}	7.4	6.6	13.2 ± 2.2	10.5 ± 1.4	NS	0.33
Lactoferrin	µg min^{-1}	0.32	0.26	0.38 ± 0.02	0.33 ± 0.03	NS	0.13
Cystatin S	AU min^{-1}	0.40	0.28	1.00 ± 0.11	0.80 ± 0.12	NS	0.22
S-IgA	µg min^{-1}	106	79	107 ± 3	82 ± 3	26%*	<0.001
Amylase activity	U min^{-1}	12.0	9.9	17.7 ± 1.5	12.8 ± 1.0	32%*	0.008
Chitinase activity	mU min^{-1}	7.7	5.1	12.3 ± 1.1	8.3 ± 1.0	37%*	0.006
Protease activity	AU min^{-1}	15.9	14.5	17.6 ± 0.9	16.5 ± 1.1	NS	0.45
Lysozyme activity	U min^{-1}	424	576	589 ± 46	696 ± 54	NS	0.13

[1] SE - standard error.

* Distributions significantly different, with mean higher in males.

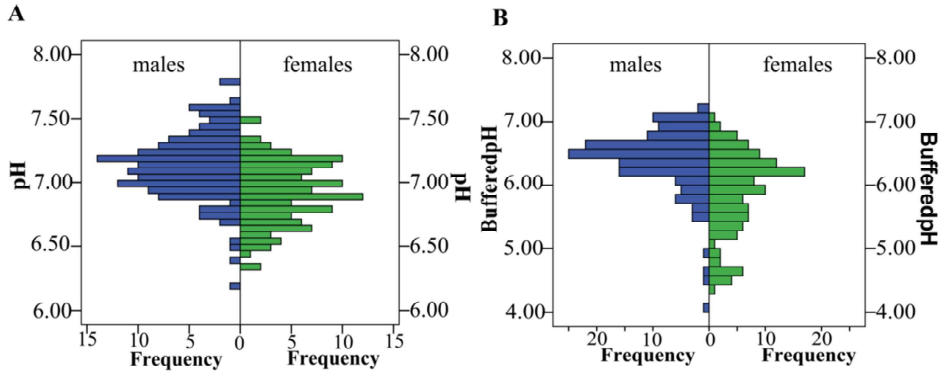

Figure 3. Distribution histograms of salivary pH (A) and buffered pH (B) divided according to sex. Males are represented in blue on the left side, females in green on the right side of both histograms. The correlation between pH and buffered pH values was $\rho = 0.83$, $p < 0.001$.

The Spectral Clustering co-occurrence plot in Fig. 4 offers an intuitive visualization of the clustering of the participants based on salivary biochemistry. Fig. 5 shows a general overview of the variation of each salivary biochemical variable across the sample population. Fig. 5 connects the clustering with the biochemical variation and provides an indication of the salivary biochemical variables that are mainly responsible for the clustering seen in Fig. 4.

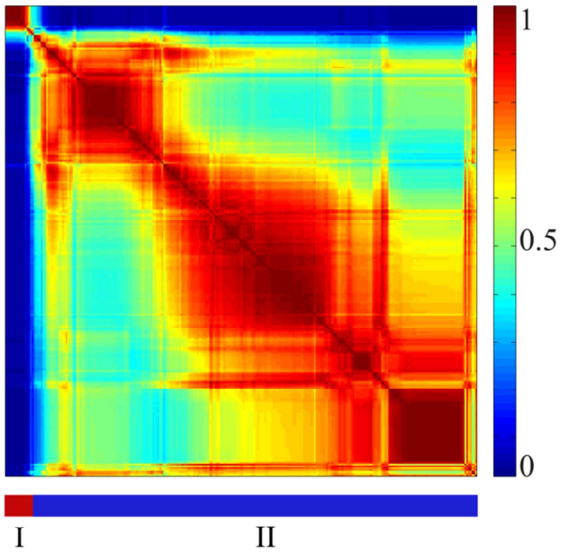

Figure 4. Spectral Clustering co-occurrence plot. Participants are ordered along both X- and Y-axis according to the co-occurrence matrix (i.e. the more similar the salivary biochemistry profiles of any two participants, the higher their tendency to cluster together and the closer they are placed on the axis). Co-occurrence values range from 0 (for participants who never cluster together) to 1.0 (for participants who always cluster together). The horizontal bar delimits the two clusters (I – red and II - blue).

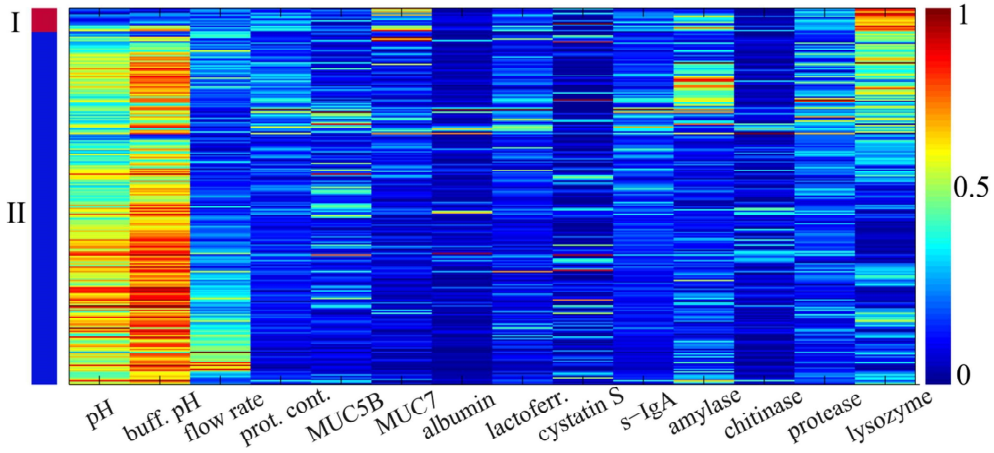

Figure 5. Visual overview of the biochemical assay results and their co-variation within the study population. The X-axis contains the 14 biochemical variables. The Y-axis contains

266 participants, arranged according to the co-occurrence matrix (same as in Fig. 3). Results for each individual assay are scaled to equal range (from 0 to 1) and color coded (legend on the right side of the figure). The vertical bar on the left delimits the two clusters (I – red and II - blue).

The first, small cluster visible in the upper-left corner of Fig. 4 (cluster I) contained 22 participants (8.3% of total number of participants, 9 males, 13 females). Fig. 5 shows that this cluster was differentiated by low pH and buffered pH values together with high lysozyme activity and MUC7 content compared to the remainder of the population. The inter-cluster differences observed in Fig. 4 were confirmed by Mann-Whitney U tests with $p < 0.001$ for all 4 biochemical variables. The participants accounting for the remaining 91.7% of the sample population made up a large cluster (cluster II, lower-right side of Fig. 3), within which 3 weakly differentiated sub-clusters could be discerned. The 1st sub-cluster (descending down the main diagonal, top-left to bottom-right) tends to group participants with average pH, high buffered pH, high amylase and lysozyme activity; the 2nd sub-cluster participants with average pH and buffered pH and low flow rate; the 3rd sub-cluster, participants with high pH and buffered pH, high flow rate and low protein content. However, these 3 sub-clusters were not sufficiently differentiated to allow for rigorous subject assignment and statistical comparisons.

Discussion

The purpose of this study was to obtain an overview of the distribution and interrelations of a set of salivary biochemical variables with known relevance for oral health, measured under strictly defined conditions in systemically healthy young adults. The participants were screened and included in case of absence of apparent caries lesions and periodontal disease. They were non-smokers. The rationale was to obtain accurate reference values of the biochemistry of saliva associated with oral health or, in other words, to construct a profile of the salivary biochemical network at a state of homeostasis. Another goal was to detect possible subgroups of individuals in the sample population based on their salivary biochemistry.

The means of the distributions of biochemical variables measured in the present study are generally within the range of values reported in previous studies, where similar conditions and analytical methods were employed [5, 15, 29-31]. Several previous investigations have measured various smaller subsets of these biochemical variables. However, methodological issues such as broadly defined study populations, diverse saliva sampling methods and different analytical methods and measurement units interfere with compiling the results across different studies. In some cases, the results are not comparable, for example where previous studies have measured the amount of a particular enzyme antigen present in saliva rather than the respective enzyme activity [32]. In other cases, differences in inclusion criteria, saliva collection and/or processing protocols may account for diverging results. This underlines the advantage of investigating a wider array of functional salivary proteins and enzymes within the framework of a single study in order to circumvent methodological bias and obtain a reliable overview.

Significant correlations were found between salivary biochemical variables. Total protein content and salivary flow rate were negatively correlated ($\rho = -0.47$, $p < 0.001$). Most of the salivary proteins and enzymes quantified correlated positively with total protein content and negatively with flow rate, although the size of these correlations varied considerably (Table 1). These findings were partly in line with results from a previous study by RUDNEY *et al.* that focused on a subset of salivary antimicrobial proteins (secretory-IgA, lactoferrin and lysozyme) [15]. The present data support their finding that secretory-IgA and lactoferrin correlate highly with salivary protein content. However, the size of the correlation observed for lysozyme was much smaller compared to the one reported in the aforementioned study, with a correlation coefficient of 0.32 as opposed to 0.62. This may be explained by the fact that for the present study lysozyme activity was measured, while RUDNEY *et al.* assessed the amount of lysozyme antigen using an ELISA technique. Also, their addition of ethylenediaminetetraacetic acid (ETDA) to the samples may have affected the results, as suggested by the authors themselves [15].

Quantifying the cumulative anti-microbial effect of lysozyme is complicated by the fact that besides muramidase activity (which kills bacteria by lysing cell walls) lysozyme also displays a non-enzymatic killing effect attributed to the activation of bacterial autolysins [33]. Interestingly, a negative correlation was found between lysozyme activity and salivary pH (ρ = -0.44, $p < 0.001$, Fig. 2). Since pH was controlled in the lysozyme enzymatic activity assay used in the present study, differences in salivary pH could not have influenced the assay results. The size of the correlation did not change after controlling for flow rate and protein content, thereby adjusting for the possibility of a confounding influence of these two factors. The inverse correlation between pH and lysozyme was mirrored in the cluster structure seen in Fig. 4 and Fig 5, with cluster I differentiated by low pH and high lysozyme activity compared to the relatively high pH and low lysozyme activity of cluster II. The mechanism behind this correlation remains unclear. It may be linked to the relative contribution of the different salivary glands to total salivary secretion. Sublingual saliva contains higher levels of lysozyme compared to parotid saliva and has a lower pH [34]. A higher contribution of sublingual saliva relative to parotid saliva would therefore increase lysozyme activity while slightly decreasing salivary pH.

Another interesting relationship was the negative correlation between lysozyme activity and chitinase activity (ρ = -0.46, $p < 0.001$). These two enzymes have somewhat complementary roles: while lysozyme cleaves bacterial cell walls, chitinase lyses fungal walls and can act against the oral pathogen *Candida albicans* [35]. The inverse relationship might suggest that these enzymes act together and that the effects of the two are cumulative, whereby a decrease in the level of one could be compensated by a concomitant increase of the other.

Significant differences between sexes were found for several of the biochemical variables examined (Tables 3 and 4). Although the mean salivary flow rate was lower in females than in males, the difference in flow rate between the two genders did not reach statistical significance, in contrast with several other studies [2, 36, 37]. Strikingly, the salivary pH distribution in females was significantly shifted towards a more acidic pH as compared to males. A similar shift towards lower values was seen for buffered pH, together with a higher number of females with extreme low buffered pH values (Fig. 3). Females made up 79% of the group of 19 participants with a buffered pH below 5.0, with the aforementioned group showing an interesting clustering effect around a buffered pH of 4.7 – 4.8 (Fig. 3). This convergence could imply that within this group of participants the first two salivary buffering systems (carbonate and phosphate) were insufficient, leading to the protein buffering system to act as a last resort. Protein buffering is the only buffering system present in saliva that is effective at pH values below 5.0 [38]. To our knowledge, sex-related salivary pH differences have not been previously reported, although lower buffering capacity in females has been recorded [39, 40]. Many studies have found higher caries prevalence in females [41, 42]. The causative factors most often invoked are social and family roles, dietary habits, genetic variations, hormonal fluctuations and lower

salivary flow rates [41, 42]. In the light of the results of the present study, lower salivary pH and buffering capacity may also be taken into consideration as potential contributors to this higher caries susceptibility.

The observed sex-related biochemical differences in saliva could be explained by physiological factors, primarily the influence of sex hormones on the salivary glands. Firstly, females have smaller salivary glands, which may contribute to the difference in salivary biochemical profiles [37]. Secondly, different gene expression profiles have been found in female salivary gland tissue as compared to males, including the expression of several genes associated with saliva secretion. This presumably is a consequence of the action of estrogen on the salivary glands estrogen receptors [43, 44]. Salivary gland sexual dimorphism and diverging gene expression profiles could explain the differences seen in salivary pH and protein composition, although the limitations of the present observational study prevent it from pinpointing definitive causative factors.

In summary, this study gives a detailed overview of the distribution and interrelations of a set of salivary biochemical variables relevant to oral health and highlights some significant biochemical differences between male and female salivary biochemistry. These findings may help advance our understanding of saliva as a functional biochemical network and its relationship to oral health.

Acknowledgements

The project was funded by TI Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. We wish to thank Nienke Hennequin-Hoenderdos, Dagmar Else Slot and Eveline van der Sluijs for their excellent contribution in developing the clinical protocol, and for coordinating and monitoring the clinical part of this study, Kamran Nazmi for his expert assistance in optimizing the biochemical assays, as well as Floris Bikker and Jan Bolscher for kindly providing protease substrates and antibodies.

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